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ENSEMBLE RECORDINGS IN AWAKE RATS: ACHIEVING BEHAVIORAL REGULARITY DURING MULTIMODAL STIMULUS PROCESSING AND DISCRIMINATIVE LEARNING

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To meet an increasing need to examine the neurophysiological underpinnings of behavior in rats, we developed a behavioral system for studying sensory processing, attention and discrimination learning in rats while recording firing patterns of neurons in one or more brain areas of interest. Because neuronal activity is sensitive to variations in behavior which may confound the identification of neural correlates, a specific aim of the study was to allow rats to sample sensory stimuli under conditions of strong behavioral regularity. Our behavioral system allows multimodal stimulus presentation and is coupled to modules for delivering reinforcement, simultaneous monitoring of behavior and recording of ensembles of well isolated single neurons. Using training protocols for simple and compound discrimination, we validated the behavioral system with a group of 4 rats. Within these tasks, a majority of medial prefrontal neurons showed significant firing-rate changes correlated to one or more trial events that could not be explained from significant variation in head position. Thus, ensemble recordings can be combined with discriminative learning tasks under conditions of strong behavioral regularity.

Key words: attention, prefrontal cortex, electrode, single unit, spike, olfactory, visual discrimination

Traditionally, the cognitive neuroscience of sensory processing and attention has mainly focused on studies in humans (Debert, Matos, & McIlvane, 2007; Hopfinger, Buonocore, & Mangun, 2000; Macaluso, Frith, & Driver, 2001; Talsma, Doty, & Woldorff, 2007) and monkeys (Everling, Tinsley, Gaffan, & Duncan, 2006; Sugihara, Diltz, Averbeck, & Romanski, 2006). There is an increasing need, however, to investigate the neural basis of these processes also in smaller vertebrates, such as rats and mice. Invasive electrophysiological recording methodology for rodents has been developed to an advanced level, such that currently tens to more than one hundred single units can be recorded simultaneously in freely moving animals (Gray, Maldonado, Wilson, & McNaughton, 1995; McNaughton, O'Keefe, & Barnes, 1983; O'Keefe & Recce, 1993; Wilson & McNaughton, 1993). To decrease the ethical burden associated with invasive primate research and take advantage of the technological and genetic opportunities in behaving rodents, we sought to develop a behavioral setup for investigating neurophysiological correlates of cognitive processes that depend on sensory processing in rats that are allowed free movement within a behavioral cage, but can also display strong behavioral regularity during stimulus sampling. We define *behavioral regularity* as stereotyped behavioral topography during the presentation of stimuli that it is required to distinguish. Achieving behavioral regularity is important not only for a precise application of stimuli, but also to assess whether changes in neural response patterns are related to cognitive processes or to motor confounds. In addition to studying sensory processing, such a setup is

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useful for exploring neural correlates of a wide variety of processes, such as stimulus discrimination learning, memory consolidation, integration of multimodal sensory information, working memory, attention, decision-making and sensorimotor control.

In primates, it has been feasible to study neurophysiological correlates of attention by reducing motor or sensory confounds during the relevant period of information processing. Usually, body, head and eye positions remain stationary during the presentation of sensory stimuli, and sensory input can be kept constant while attentional demands are being varied (e.g., Steinmetz, et al., 2000; Treue & Maunsell, 1996). This stasis can be achieved using head fixation by skull-implanted head bolts and other measures such as continuous eye tracking. We sought to achieve behavioral regularity in freely moving rodents to study neural correlates of cognitive processes without marked sensorimotor confounds.

Much progress has been made in developing behavioral paradigms to test sustained or divided attention, recognition memory, working memory, attentional set shifting and many other tasks in rodents (Birrell & Brown, 2000; Brigman, Bussey, Saksida, & Rothblat, 2005; McGaughy, Turchi, & Sarter, 1994; Muir, 1996; Robbins, 2002; Sarter & McGaughy, 1998; Tse, et al., 2007), but often profound adaptations of these tasks are necessary when motor or sensory confounds must be minimized, such as in unit recording studies. In contrast, suitable behavioral methodology has been developed to examine the neurophysiological processing of unimodal sensory stimuli (Polley, Steinberg, & Merzenich, 2006; Szabo-Salfay, et al., 2001) and discriminative learning within a single sensory modality (Schoenbaum, Chiba, & Gallagher, 1999; van Duuren, et al., 2007), but also this field of research may benefit further from novel equipment allowing stronger control over and monitoring of behavior and simultaneous, time-controlled application of stimuli across multiple sensory modalities in freely moving rats.

To address this issue, we designed a multimodal stimulus chamber (MMSC) and surrounding behavioral cage to meet the following requirements: (i) it should allow the animal to display a stereotyped, regular behavior and body posture during stimulus sampling, at least for a restricted period of time; (ii) stimuli should be presented to the animal in an automated and time-controlled manner, in at least two sensory dimensions (visual and olfactory); (iii) the MMSC and surrounding behavioral cage have to be compatible with sizable headstages for independent positioning of multiple electrodes and chronic recordings in targeted brain areas; (iv) the cage should offer sufficient means to assess behaviorally whether the animal performs a sensory or cognitive task correctly or not, that is, it should comprise a subsystem allowing the animal to behave and be reinforced appropriately. Instead of offering a solid, multidimensional object for the animal to explore with many degrees of motor variability, we chose the solution of essentially creating a ''hollow'' object (i.e., the MMSC) which can be explored in a time-controlled manner by the rat making head entries into it. In this article we describe the MMSC system and training procedures used to produce behavioral regularity in discrimination tasks so that aspects of stimulus control and behavior can be clearly related to firing of neurons in freely moving rats. We validate the system by successfully training rats in it on a sensory discrimination task, showing behavioral disruption and adjustment when a second, distractive set of stimuli from another sensory modality is introduced.

METHOD

Subjects

Before the onset of experiments, male Lister-Hooded rats (N = 4; Harlan, the Netherlands; body weight 250 g) were allowed to acclimatize for one week in a 12-hr light/12-hr dark cycle (light on 08:00) and were housed in pairs. Once the experiment started, rats were housed solitarily. Food (Harlan Teklad, Global 18% Protein Rodent Diet) was available ad libitum. Animals had access to a water bottle for approximately 0.5 to 1.5 hr after the end of a behavioral session. All experiments were carried out in accordance with national guidelines on animal experimentation and were conducted in a room dimly lit with orange lights.

Apparatus

Multimodal stimulus setup. The multimodal stimulus setup consisted of three subsystems

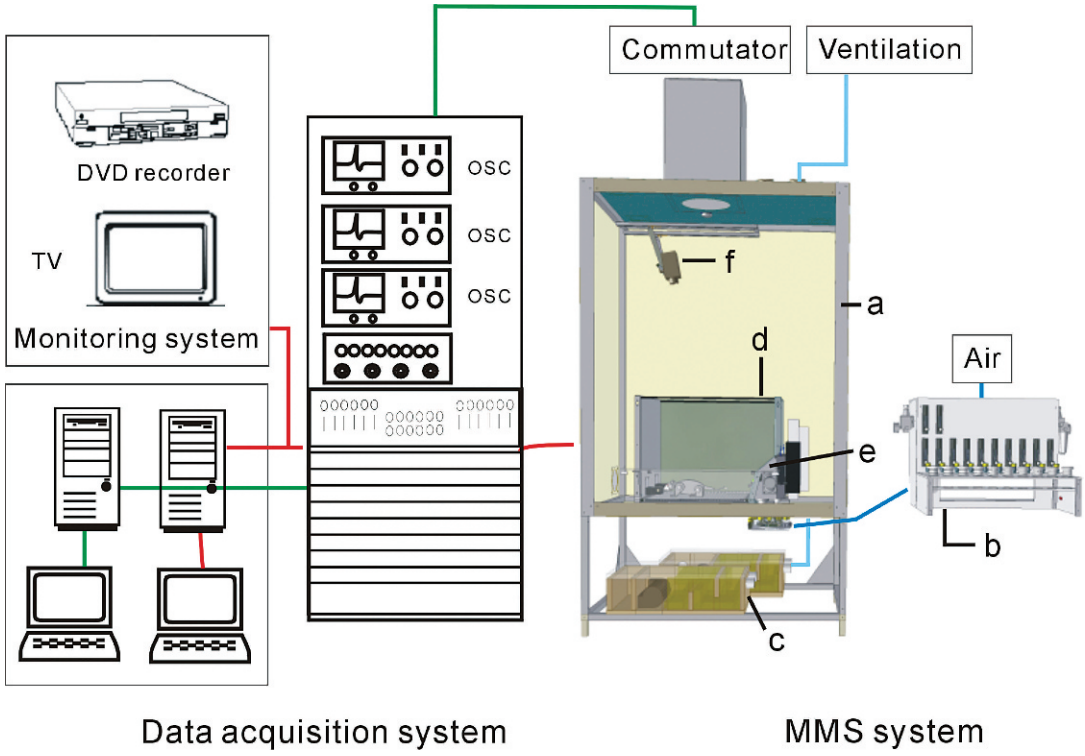

Fig. 1. Behavioral system for multimodal stimulus presentation and discrimination learning. The data acquisition system, comprising amplifiers, oscilloscopes (OSC) and a behavioral monitoring and recording system, is shown on the left. The multimodal stimulus system is shown on the right and includes a Faraday cage (a), an odor application system (b), a DC-powered ventilator (c), a behavioral cage (d) with attached multimodal stimulus chamber (MMSC) (e). A videocamera (f) was attached to the ceiling of the Faraday cage and, upon neurophysiological recording, spike and EEG signals were conveyed to the amplifiers via a headstage, cables and a commutator.

(see Figure 1): (i) a behavioral cage, the MMSC, stimulus delivery facilities and a system for commanding this behavioral setup, being installed on a personal computer and using a Rabbit 2000 microprocessor (type RCM2250, Delmation Products, Zoetermeer, the Netherlands); (ii) a behavioral monitoring system, comprising a videocamera (Cohu2200; Cohu Inc., San Diego, U.S.A.), a videotracker for tracing the animal's head position (Neuralynx, Bozeman MT, U.S.A.), a TV monitor and DVD recorder; (iii) an electrophysiological data acquisition system.

The larger behavioral cage (51.6 × 30.0 × 39.6 cm; Figure 2A) contained a grid floor and a fluid well. Both the MMSC and behavioral cage were placed inside a Faraday cage (Figure 1A; 100 × 75 × 125 cm, covered with sound-attenuating material). The MMSC and adjacent behavioral cage were separated by a wall containing a head-entry port (Figure 2A). The videocamera and a house light were mounted on one of the inside walls of this Faraday cage. To avoid interference of video-tracked rat positions by light reflections, all parts of the behavioral cage exposed to the camera were made of dull black materials. The fluid well was modified after a design by Schoenbaum and Setlow (2001); its gravity-fed fluid supply system contained four lines, each operated by a solenoid valve (Versa valve, E5SM series, Doedijns, Cuijk, the Netherlands), three of which delivered fluid to the well (sucrose, quinine or water to flush the lines) and one controlled suction. Onsets and offsets of nose pokes into the fluid well were detected using an LED detector. In addition, we measured onset and duration of licking behavior by an optic detector (based on type: Banner, D12DAB6FP AC-coupled; Clearwater Technologies, Boise ID, U.S.A.). The wall panel with head-entry port and trial onset

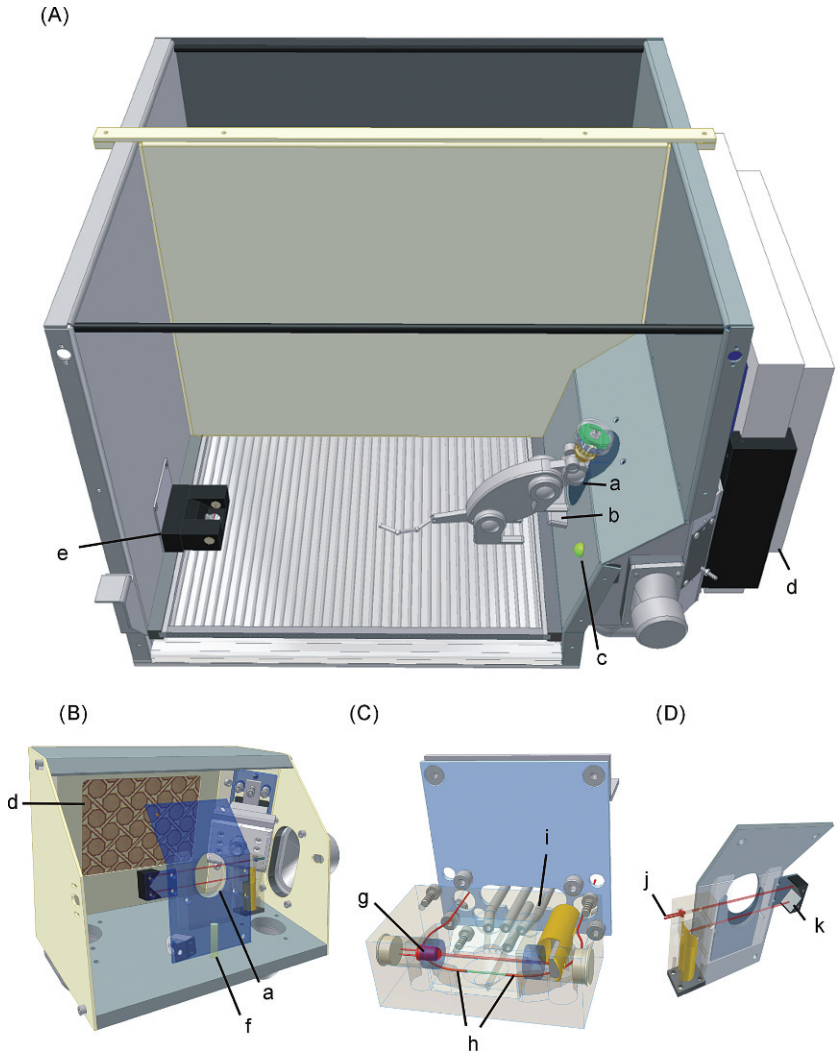

Fig. 2. Details of the multimodal stimulus chamber (MMSC) and adjacent behavioral cage. A: the behavioral cage included a head-entry port (a) for gaining access to the MMSC; a horizontal shelf upon which the rat put its forepaws during stimulus sampling (b); a light for signalling trial onset (c); an LCD screen for presenting visual stimuli (d); and a fluid well (e). B: MMSC, with head-entry port (a); LCD screen (d); and odor delivery nozzle (f). C: fluid well, with LED detecting ''nose down'' response (g); optic sensor detecting licking behavior (h); and three nozzles for fluid delivery (one of which is indicated by 'i'). D: front panel of the MMSC with head-entry port fitted with LED (j) and a mirror (k) for creating a dual beam, facilitating detection of head entry.

light was situated on the opposite side of the behavioral cage (Figure 2A). To promote stasis of the rat's head and body position during stimulus sampling, the head-entry port was placed at a relatively elevated position above the grid floor (center point: 9.5 cm above floor; diameter 3.0 cm) and a shelf ($2 \times 7 \times 0.7$ cm) was installed onto the wall, 4.8 cm below the center of the port. In practice, rats easily learned to place their forepaws onto the shelf while poking into the port. Both the MMSC and surrounding behavioral cage were commanded and monitored by the Rabbit 2000 microprocessor system; software for behavioral control was written in Dynamic C and Visual C++.

Trial onset was marked by lighting a green LED on the right side of the head-entry port (Figure 2A). To detect head entry and withdrawal, we constructed a dual-beam infrared

light detector (Figure 2D; Farnell, Leeds UK, Sharp photodetector, type 970-7840) using a set of two mirrors 90° angled to each other. The MMSC contained two air-pipes (for removing odor stimuli), a speaker and a microphone for presenting and detecting sound stimuli (details of which will not be presented here) and a 43.2-cm (17 inch) flat monitor for visual stimuli (Figure 2B). The odor application and removal system was based on a design by Schoenbaum that used vacuum suction (Schoenbaum, 2002), but we used ventilators in addition to vacuum lines to quickly remove large-volume odor remnants from the MMSC. A custom-made camera with telescopic lens was placed inside the MMSC for visual inspection of the rat's eye and head position inside the MMSC. The computer screen displaying visual stimuli was placed opposite to the wall segregating the MMSC from the larger behavioral cage (Figure 2B), so that the rat was facing the screen upon head entry at a distance of approximately 14 cm. While a large part of the screen was covered by a wall plate, the visual stimuli were presented through a transparent, plexiglass window in this plate (12 × 9 cm) to prevent leakage of odor out of the MMSC.

Odor application system and control of stimulus timing. To achieve optimal timing of odor application, we set up an airflow containing a preselected odor already well in advance of stimulus onset, routing this airflow through a bypass until the moment of odor presentation in the MMSC. First, odorized air, collected from each of nine glass vials containing fragrance odorant oil (Tokos B.V., Noordscheschut, the Netherlands), was mixed in a 1:1 ratio with clean air pumped in via a pressure line. At a flow rate of 1.5 l/min, this mixture was conducted to an odor-selection station composed of 10 solenoid valves (type ET-2M-12V DC, Clippard Instruments, Cincinnati OH, U.S.A.). During the intertrial interval (ITI) an odor presentation was prepared by opening a series of valves that routed the odorized air flow via a bypass unit into an exhaust line operated by a modified DC-powered ventilator (motor type: AXH 230 KC-A, Oriental Motor Co., Torrance CA, U.S.A; fan type: Cross-Flow Blower, TAS18B-002, Trial S.P.A, Italy; capacity 3.0 l/min.; valve 2 ''bypass unit'' and switch 4 ''fan out'', respectively). Meanwhile, the air flow was prevented from entering the MMSC by keeping two other valves closed (valves 1 and 3). Both during trials and ITIs, operation of the exhaust ventilator kept the MMSC under negative pressure to avoid possible leakage of odor into the behavioral cage. Once the rat poked his head into the chamber, the exhaust ventilation was turned off (switch 4 at ''fan out'' closed) and 300 ms following nose poke onset, the odorized air was routed into the MMSC by opening valve 1 and closing valve 2, while valve 3 remained closed. Following stimulus sampling (>700 ms), odorized air was removed by activating a vacuum line (valve 3; −5 kPa) as well as the bypass route again (valve 2 open and switch 4 on), whereas valve 1 was closed. Following head withdrawal and fluid sampling, the valve controlling vacuum suction (valve 3) was closed again and the odor controlling system was returned to ITI state.

For fast presentation of visual stimuli, the computer was programmed to retrieve the appropriate file from a multimedia event list during the ITI. During the ITI the visual pattern remained occluded by a black screen (''mask''), so that the visual stimulus was retrieved from memory and prepared for presentation, but not yet presented to the rat. The latency between the computer command and onset of the visual stimulus was less than 10 ms. This method of presentation was faster by about 260 ms and more reliable than when the visual stimulus had to be retrieved from memory upon stimulus presentation. After at least 300 ms had elapsed following head entry into the MMSC, the black screen was removed; it was reinstated again after the stimulus sampling period (>700 ms) was over.

Procedure

General aspects of behavioral tasks. Although various types of task were employed, the behavioral setup will be explained according to the structure of the most basic task used, a simple discrimination (SD) task. The onset of a trial was marked by a trial light turning on (Figures 2A and 3); a trained animal subsequently poked its head into the entry port (Figure 2A) and thereby gained access to the MMSC (Figure 2A, B). Once its head was stationary inside this chamber, a visual or olfactory stimulus was presented for 700 ms. Each stimulus belonged to a pair of stimuli within the same sensory modality, one of

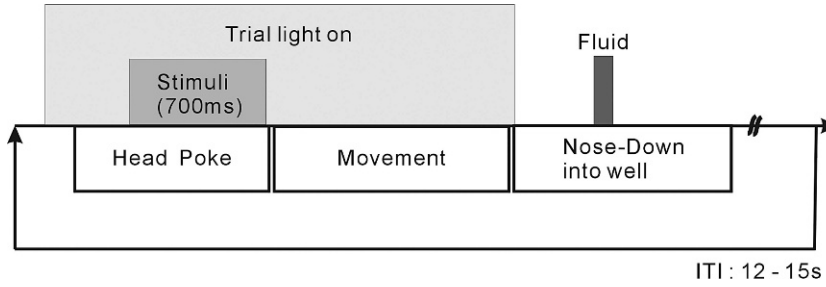

Fig. 3. General time schedule of a trial for both simple and compound discrimination. A trial was initiated by the onset of a trial light. Upon a head poke by the animal into the MMSC, a single unimodal stimulus was applied (SD) or two stimuli of different modality were simultaneously presented (CD). Upon head withdrawal from the MMSC, the rat generated either a NoGo or Go response. In case of a Go response, the rat walked over to the fluid well (movement period), put its nose down into this well and consumed a volume of sucrose or quinine solution. Trials were separated by an intertrial interval.

which (S+, the positive stimulus) was coupled to reward if the animal performed a correct ('Go') response (150 µl sucrose solution, 0.3 M in distilled water; Merck) and the other (S−, the negative stimulus) to an aversive stimulus that punished 'Go' responses to this stimulus (150 µl quinine solution, 0.02 M in distilled water; Sigma). The animal learned to generate a Go response following an S+ (''hit'') and a NoGo response following an S− (''correct rejection''). Following stimulus delivery and head retraction from the port, the Go response consisted of a locomotor response to the fluid well (Figures 2 and 3), and an additional ''nose down'' response into the fluid well, which was required to last at least 500 ms before fluid was delivered. The rationale for implementing the locomotor, or movement, period was twofold. First, it offered an opportunity to consider movement response latency as an additional measure of learning (Figure 6). Second, in previous studies we found interesting neural correlates of reward expectancy specifically during this trial period (Van Duuren et al., 2007). When these actions were either omitted or the rat failed to visit the fluid site within 5 s, performance was classified as a correct rejection (for the S−) or a ''miss'' (failure to Go following an S+). A ''false alarm'' response was scored when the rat made an erroneous Go response following an S−. After a reinforcer was delivered to the well, the rat was allowed to consume it within 8 s, after which a vacuum line was activated to remove the fluid, and water was directly flushed in and out again to clean the tray. The duration of the ITI ranged from 12 to 15 s and was selected pseudorandomly. More complicated behavioral tasks included multimodal compound discrimination (see ''fifth phase'' of training below).

Behavioral training. Prior to the main experiment, the rat went through five pretraining ('shaping') phases, including habituation to the behavioral cage. In the first phase (one session, 15 min) every head poke into the MMSC, followed by a nose down into the fluid well, was rewarded with sucrose solution. In the second phase (2–5 sessions, 50 trials per session), the animal was required to keep its head in the stimulus port for a period that varied from 500 ms in early sessions to 1000 ms in later sessions in order to receive a reward. In the third phase (1–2 sessions, 80 trials per session), upon head entry for at least 300 ms, a visual or odor stimulus was presented for 700 ms. Reward was delivered when the rat sustained his head poke for at least 1000 ms and subsequently moved to and kept its nose in the fluid well for at least 500 ms. If the rat retracted his snout from the well before 500 ms had elapsed, no reward was delivered and a new trial was initiated.

In the fourth phase (6–14 sessions, 80 to 112 trials per session), one of two stimuli from a single modality was presented, with visual stimuli in the initial sessions and olfactory cues in the latter sessions. A ''Go'' response was reinforced with sucrose following the S+; a Go response led to delivery of quinine solution following the S−. S+ and S− trials were presented pseudorandomly in a 1:1 ratio. Each session contained several blocks, each composed of eight S+ and eight S− trials. Rats

were required to make at least 70% correct rejections on the S− trials for at least two consecutive blocks of trials (cf. Garner, et al., 2006). This criterion was based on correct rejections because, in general, the rats showed a much stronger tendency to perform Go responses than to withhold these. When the rat met the criterion for two consecutive sessions, it was trained on a novel set of two exemplars in each of the two sensory dimensions. Thus, by the end of the fourth phase the rat had been trained on a total of four exemplar sets, two in each dimension.

In the fifth phase, the rat was first trained on a continued SD schedule to distinguish two exemplars that had been used in a previous training phase, viz. as the first stimulus set used within the same modality as currently applied. When the criterion was met again, compound discrimination (CD) was introduced: in addition to the modality previously used for SD, new examplars from a second modality were presented synchronously with the same exemplars from the first modality. The newly added modality was the irrelevant dimension and thus conveyed no predictive power about which stimulus in the other modality would be followed by reward or punishment in case of a Go response. Each of the two exemplars from the relevant dimension was co-presented with each of the exemplars from the irrelevant dimension (Figure 4). Across sessions, the number of blocks gradually increased from seven to nine, resulting in a total of 144 trials per session.

The video clip that is included as a supplement to the online version of this article shows a typical rat's behavior in two consecutive trials. The clip starts with a Go trial and moves on to a NoGo trial in a compound discrimination task.

Multi-electrode array, surgery and data acquisition. After pretraining, the animals (body weight : 400–460 g at time of surgery) underwent surgery and implantation of a tetrode recording array (''hyperdrive''; Gothard, Skaggs, Moore, & McNaughton, 1996; Gray, et al., 1995; Lansink, et al., 2007). A tetrode is a microbundle of four tiny electrode wires (each about 13 μm in diameter) twisted together (Gray, et al., 1995; Lansink, et al., 2007; McNaughton, et al., 1983; O'Keefe & Recce, 1993; van Duuren, et al., 2007). The array contained 12 tetrodes, two reference electrodes, each with a diameter of about 25 μm, and two extra electrodes for recording EEG (a twisted pair of Teflon-coated stainless-steel wire, 50 μm in diameter). Tetrodes were mounted on independently movable drivers, emerging at the bottom end of the hyperdrive from a ''flat'' (i.e., roughly ellipsoid) bundle (approximate dimensions: 0.8 × 2.0 mm) and fitting into the mediolateral width of the medial prefrontal cortex.

Before surgery, the rats were given oral ampicillin (30 mg/kg, Eurovet, the Netherlands) mixed with 10% sucrose solution on a 3-day-on/2-day-off regimen. Animals were anesthetized with Hypnorm (0.06 ml/100 g body weight, i.m.; 0.2 mg/ml fentanyl and 10 mg/ml fluanison; Janssen Pharmaceutics, Beerse, Belgium) and dormicum (0.03 ml/ 100 g, s.c.; midazolam 1.0 mg/kg; Roche, Woerden, the Netherlands) and mounted in a Kopf stereotaxic frame with bregma and lambda in the horizontal plane. Surgery involved the stereotaxic implantation of the flat tetrode bundle through a rectangular craniotomy (about 2 × 3 mm) above the right medial prefrontal cortex (center point, AP: +3.0 mm, ML: as close to the sagittal sinus as possible). After removing the dura and placing the bundles flush on the cortical surface, the cortex was covered with a layer of Silastic (i.e., a biocompatible, silicone elastomere, World Precision Instruments, Berlin, Germany). One hole was drilled over the right hippocampus (AP: −3.8 mm, ML: 2.4 mm) and the extra EEG electrodes were inserted into dorsal hippocampus (DV: 3.3 mm). The hyperdrive and electrodes were kept in place with dental cement and eight anchor screws, one at the contralateral side serving as ground.

Upon recovery from anesthesia, rats were administered 0.3 ml/ 100 g of diluted Fynadine (10% in physiological saline, s.c.; Flunixinum 50 mg/ml, Schering-Plough Animal Health, Brussels, Belgium) for analgesia, and received oral doses of ampicillin (30 mg/kg) for 3 days consecutively and on a 10-day-off/10-day-on regimen for the duration of the experiment. Starting at the day of surgery, tetrodes were gradually moved down towards the prelimbic cortex across a period of 7 days. The two reference electrodes were placed in the superficial layer of the dorsal frontal cortex or anterior cingulate cortex (Fr2, ACC; Paxinos & Watson, 1998). After a week

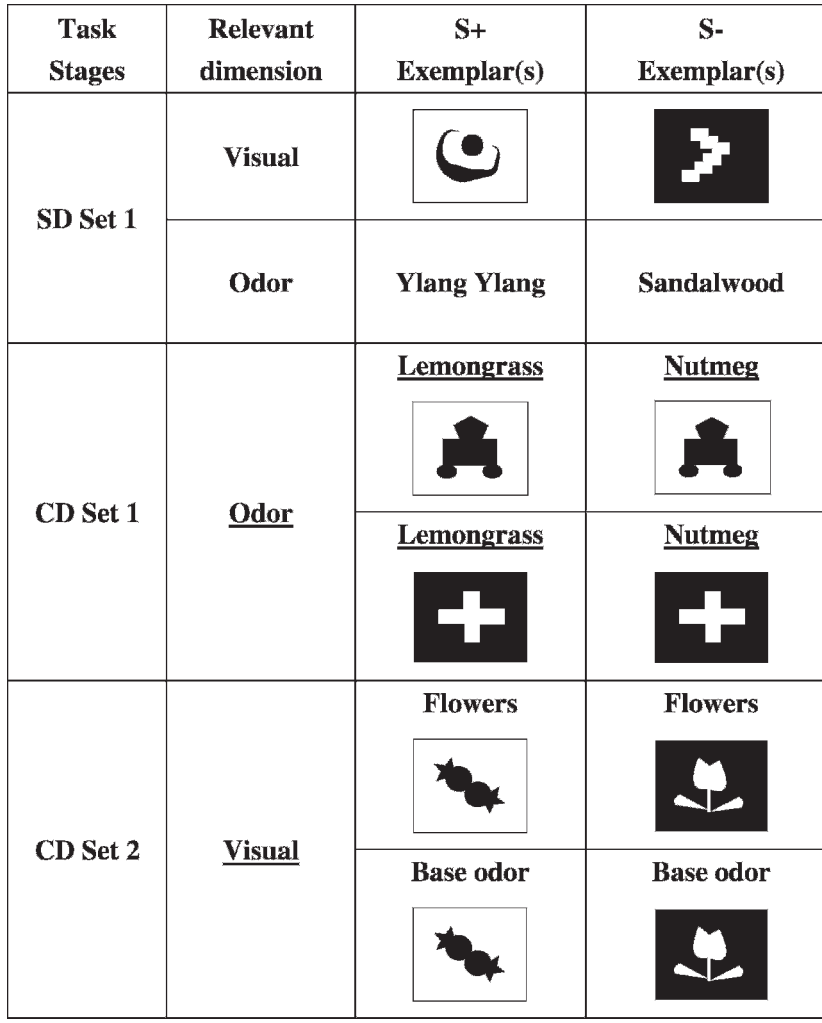

Fig. 4. Stimulus presentation schedules of the simple (SD) and compound (CD) discrimination tasks. Chronological order is from top to bottom. Four rats were trained to discriminate visual stimuli first (top row) and then proceeded with simple odor discrimination. This training was followed, first, by compound discrimination with odor as relevant dimension (using four combinations consisting of two novel odors and two novel visual patterns; CD set 1) and subsequently with vision as relevant dimension (CD set 2, using novel examplars in both the visual and olfactory domain). Note that in the CD phase, the S+ and S− were combined with exemplars in the irrelevant dimension. The 4 rats all experienced the same visual and olfactory examplars in the same order.

of recovery, the rats performed the same task as in the fifth phase of training and with the same examplars, while at the same time parallel spike and EEG recordings were performed across 64 channels.

Neuronal signals were passed through a 54-channel unity-gain amplifier headstage (Neuralynx) and amplified, filtered (5,000× and 0.6–6 kHz for spikes, 10,000× and 1–475 Hz for EEG recordings) and transmitted to the Cheetah Data Acquisition system (Neuralynx). Signals that crossed an amplitude threshold triggered a brief (1 ms) digitization at 32 kHz on all channels of the tetrode, and the spike waveforms were stored on a personal computer. A circular array of light-emitting diodes (LEDs) was mounted on the headstage to track the animal's position during behavioral recording at 25 frames/s. A behavioral-event signal, generated by the rabbit system, was delivered via a serial-to-parallel converter (type: AVR-H128, ATMega, Lelystad, the Neth-

erlands) to the TTL input port on the Analogue-Digital Interface (Neuralynx) to synchronize neural and behavioral-event data. In addition, the behavior of all rats was recorded on DVD.

Spikes were sorted off-line on the basis of the amplitude and principal components of events recorded on all four tetrode channels by means of semiautomatic and manual clustering algorithms (KlustaKwik and MClust), resulting in a spike time series for each of the isolated cells (for further details, see Lansink, et al., 2007 and van Duuren, et al., 2007).

Data Analysis

Neural data were analyzed by custom-made code and toolboxes in Matlab (MathWorks, Gouda, Netherlands). To assess correlations between neuronal firing rate and task events, we produced a smoothed peri-event time histogram (PETH) using a local regression method (Loader, 2004; ''logfic'' toolbox in open-source Chronux algorithms, http://chronux.org) after averaging across trials. The smoother was a quasi-Gaussian function with window using 0.3 fixed bandwidth. After smoothing, a two-sample Kolmogorov-Smirnov (KS) test was used to detect differences in firing patterns in trials with positive (rewarding) versus negative (punishing) outcome. Changes in firing rate during the trial period were defined as activity increments or decrements relative to baseline firing levels, which were measured in the time window from −9 to −2 s before the onset of the trial light. In order to avoid assumptions on particular spike train distributions (e.g., Poisson), we used a bootstrapping method to estimate the distribution of mean firing-rate values for each bin of the ITI. By this method the collection of spike counts per trial was randomly resampled for each time bin 1000 times with replacement, and next a 95% confidence interval in mean firing rate was calculated by using a corrected percentile method (cf. Wiest, Bentley, & Nicolelis, 2005). Only correct trials were considered.

In order to examine variation in the rat's head position during the stimulus sampling period, we used video-tracking data to calculate the mean Euclidean travel distance of the rat's head center per trial, calculated by summation of all sample-to-sample changes in head position over the relevant stimulus periods. To assess whether the rat assumed a different head position depending on the type of trial and impending response, we first applied a two-way analysis of variance (ANOVA) with trial type (hits versus correct rejections and SD versus CD) as factors, and Euclidan distance as the dependent measure. Moreover, we computed the mean X- and Y-positions of the rat's head during stimulus sampling in simple and compound discrimination sessions, as well as 95% confidence intervals around the mean using bootstrapping.

Histology

After finishing a recording experiment, small electrolytic lesions were made at the tetrode endpoints in the brain area of interest by passing current (25 uA, 10 s per lesion) through one of the leads of each tetrode. One day later rats received an overdose of Nembutal (0.2 ml/ 100 g body weight; CEVA Sante Animale, the Netherlands) and their brains were fixed through transcardial perfusion with 0.9% NaCl solution followed by 4% paraformaldehyde in 0.1 M Phosphate buffer (pH 7.0, Klinipath, the Netherlands). Brains were cut in coronal sections (40 μm) using a Vibratome (Leica, type VT-1000S, Wetzlar, Germany) and Nissl-stained.

RESULTS

Behavior

All 4 rats learned to perform the SD and CD tasks at least until criterion, although the number of sessions needed to reach criterion varied across rats (e.g. Figure 5). Learning was well monitored by tracking the percentage of correct rejections (Figure 5). Acquisition of correct rejections for individual rats and mean percentage of hits as a function of progressive visual SD sessions is plotted in Figure 5A, while Figure 5B represents acquisition of the olfactory SD task.

Response latencies (i.e., time lapsed between the rat's withdrawal from the MMSC and its nose poke into the fluid well) for hits and false alarms are depicted in Figure 6A for visual discrimination. By the ninth visual training session, the average latency for hits was significantly shorter than for false alarms (Figure 6A, $p < .05$ for sessions 9–12, paired t-

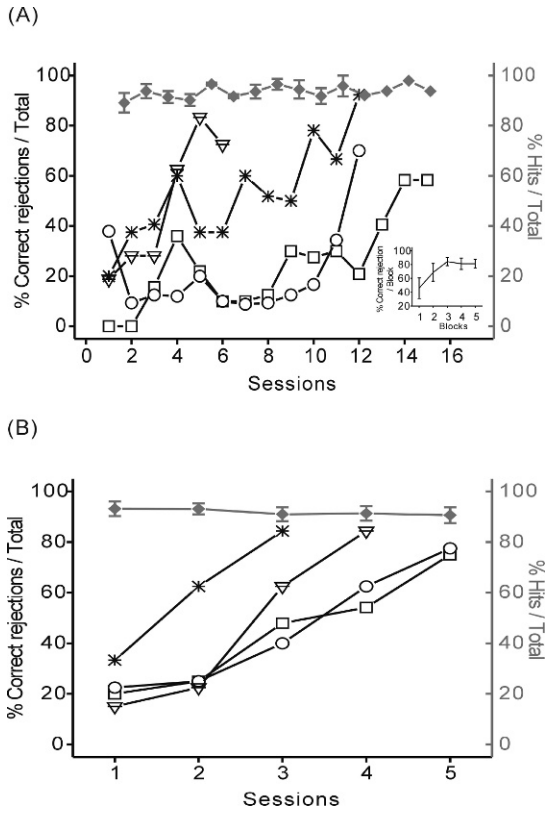

Fig. 5. Performance in simple discrimination learning. (A) The percentage of correct rejections (NoGo responses to S−) in the visual discrimination task was plotted in black as a function of session number for 4 individual rats indicated by different symbols. The mean percentage of hits (Go responses to S+) of the same rats is shown in gray. Inset shows average performance in each block of the last session in the main panel. Criterion was at 70% correct rejections in two consecutive blocks. (B) Idem for olfactory discrimination, which followed the visual task in time.

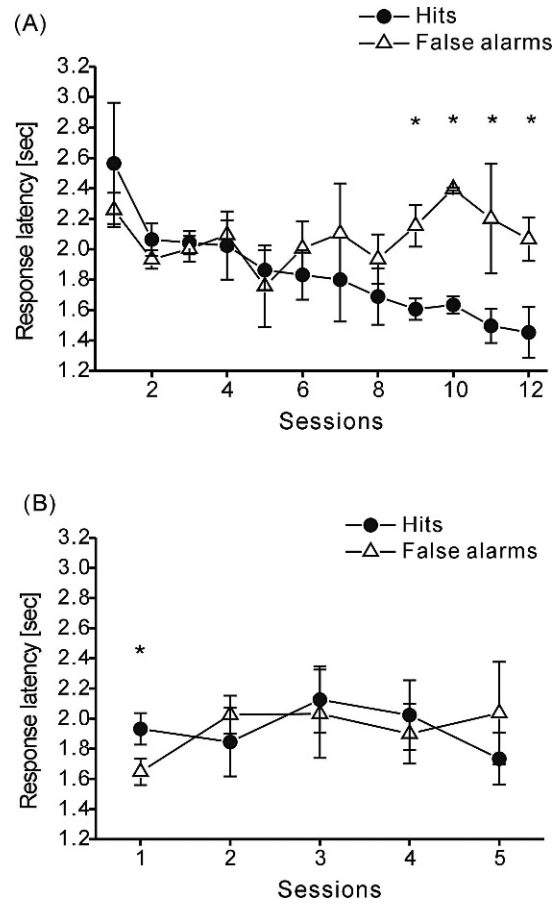

Fig. 6. Response latencies in simple discrimination learning. (A) Response latency in simple visual discrimination plotted as function of session number; open triangles symbolize mean latency for false-alarm (erroneous Go) responses, filled circles symbolize hits (correct Go responses). The mean latency was different ($p < .05$, ANOVA) for these two types of responses in the final four sessions (marked by *). (B) Idem for simple olfactory discrimination; the latency for hits and false-alarm responses differed significantly only in the first session.

test following ANOVA). Except for the initial session, the response latency differences were not significantly different for subsequent simple olfactory discrimination studied in the same 4 rats (Figure 6B), possibly because task acquisition in the olfactory dimension proceeded more quickly than with visual stimuli, $p < .05$, paired t-test. The number of sessions to reach criterion was 11.25 ± 1.89 (mean ± s.e.m.) for visual SD, and 4.25 ± 0.48 for olfactory SD.

Figure 7A illustrates correct rejections on the subsequent CD task, using odor as the relevant (i.e., outcome-predicting) dimension and vision as the irrelevant, distracting dimension. All 4 rats attained criterion performance in the first session, which was composed of an initial set of SD trials (using only odor as stimulus; trial blocks labeled SD1–SD3 in Figure 7A), followed by a switch to compound stimulation (blocks CD1–CD5) as soon as criterion was reached. The distracting visual stimulus resulted in a mild and brief decrease in performance in only one rat (change from SD3 to CD1: +3.1 ± 9.4%; n.s., N = 4).

In contrast, when the rats were trained in a simple discrimination paradigm with vision as the relevant dimension, addition of odors as irrelevant stimuli in the CD phase led to a

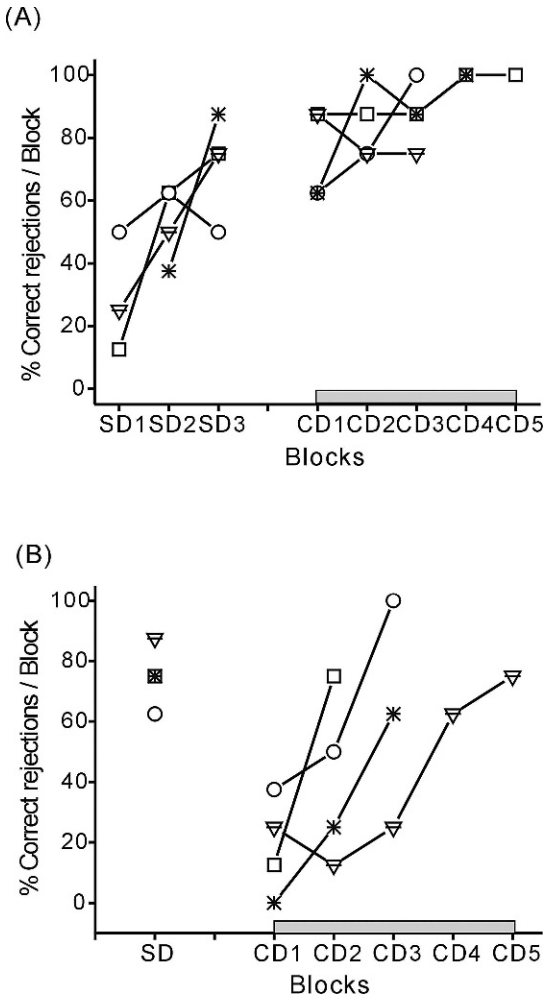

Fig. 7. Discriminative performance before and after the transition from simple to compound discrimination learning. The percentage of correct rejections is plotted as a function of trial blocks, each of which contained eight S+ trials and eight S− trials. (A) presents the transition from simple olfactory discrimination to the compound phase, where odor remained the relevant dimension. This session followed the olfactory SD task (Figure 5 and 6B) in time. In (B) rats performed simple visual discrimination and proceeded with the compound phase, keeping vision as relevant dimension. This session followed the transitional SD-to-CD olfactory task (Figure 7A) in time. See Figure 5 for plotting conventions and behavioral criterion.

strong but temporary deterioration of performance (change from SD to CD1: $-56.3 \pm 10.8\%$, $p < .05$, N = 4, paired *t*-test).

Neurophysiological Data

Two rats, both having undergone pretraining and the SD and CD tasks illustrated in Figure 5 and 7, were fitted with a microdrive containing a tetrode array converging into a flat bundle impinging upon the medial prefrontal cortex. Rats recovered within a few days after surgery, and were able to maintain head position as they did prior to surgery. Postmortem histology confirmed that the tetrodes penetrated into the medial prefrontal cortex, comprising the dorsal regions FR2 and CG1 (Paxinos & Watson, 1998) as well as prelimbic cortex.

We analyzed a total of nine recording sessions during which rats performed a visual (five sessions) or olfactory (four sessions) discrimination task. These sessions yielded a total of 301 well-isolated single units with an average of 33.4 ± 3.8 units per session. Firing patterns were analyzed by constructing smoothed PETHs synchronized to the onset of a trial event. Of these units, 196 units (65.1%) displayed responses to task events that were statistically significant relative to baseline activity. Most of these units with task correlates (60.7%, N = 119) showed firing rate increments, whereas task events correlated to decrements were observed in a remaining 39.3% of units (within a time window of −1.5 to 3.5 s relative to stimulus onset at t = 0 s). In short, all events or phases relevant for task performance were well represented in mPFC populations, including neural responses during stimulus sampling, movement, waiting and consuming fluids. Figure 8 presents two examples of single units displaying differential activity in hit and correct rejection trials during the sampling period of SD tasks. One unit showed a firing rate increment mainly at and after a late stage of stimulus presentation in the visual SD task, but also discriminated between the hit and correct rejection (Figure 8A; $p < .05$, KS test). A second unit, recorded in a different rat performing odor discrimination, showed a similar firing pattern, although the difference between the neural responses was not significant (Figure 8B; $p > .05$, KS test).

Head Movement During Stimulus Sampling

In a total of 10 sessions from the 2 rats (SD: 6 sessions, 3 with odors, 3 with vision; CD: 4 sessions) we analyzed behavioral variation during the stimulus sampling period, as assessed from changes in head position. The total head travel distance per trial did not

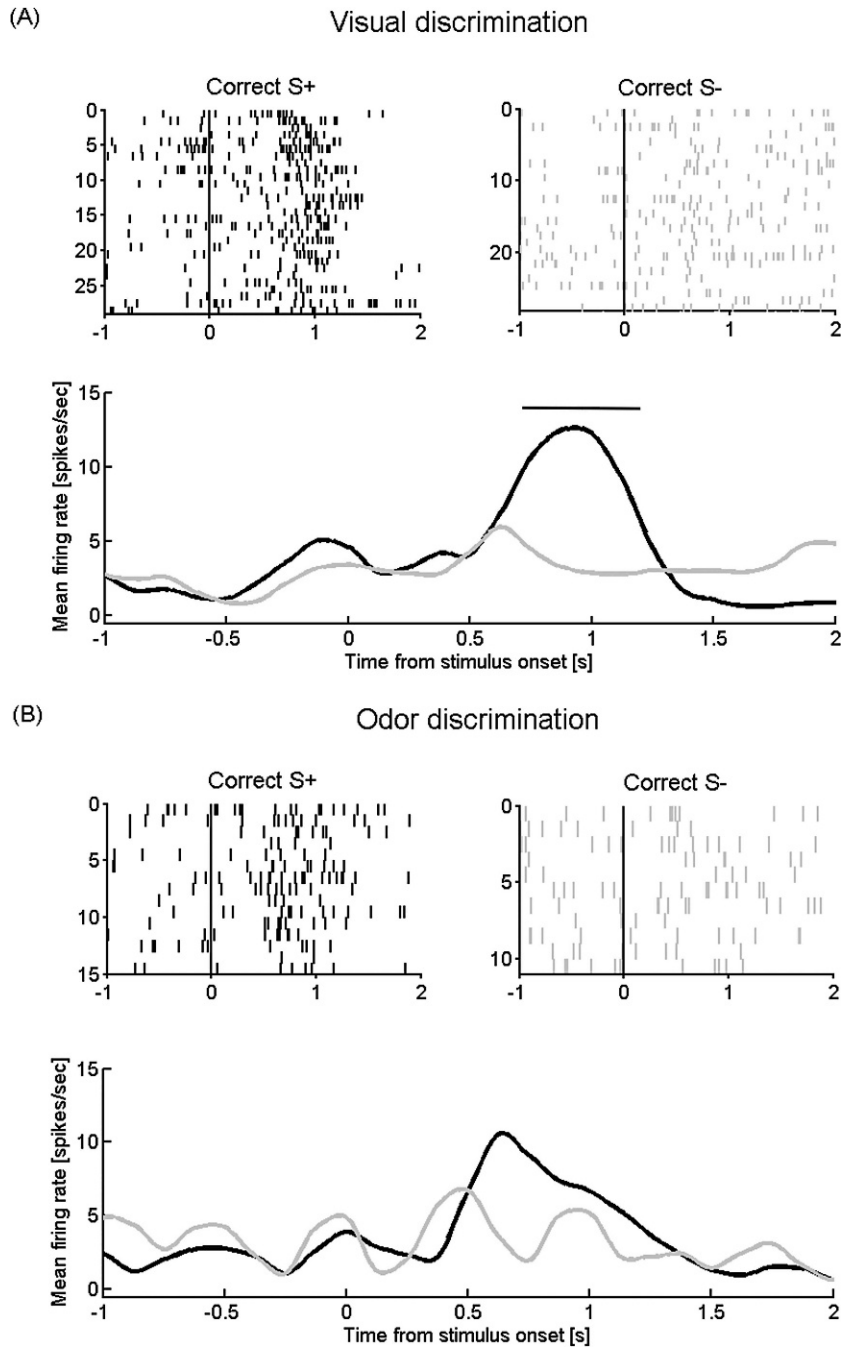

Fig. 8. Examples of peri-event time histograms (PETHs) synchronized on stimulus onset, taken from two medial prefrontal single units. (A) Raster plots of PETHs for correct responses on S+ (left) and S− (right) trials in a simple visual discrimination task. A correct response on the visual S+ consisted of a Go response towards the fluid well (outcome: sucrose solution), whereas a correct response on the S− was a NoGo response. The graph below the raster plots shows the smoothed mean firing rate for Correct S+ (black) and Correct S− (grey) trials, departing from a bin size of 50 ms. The two curves were significantly different at $p < .05$ as indicated by a horizontal bar on top of the curves. (B) Idem as (A), but now for a simple odor disrimination task. Curves for Correct S+ (black) and Correct S− (grey) did not differ significantly.

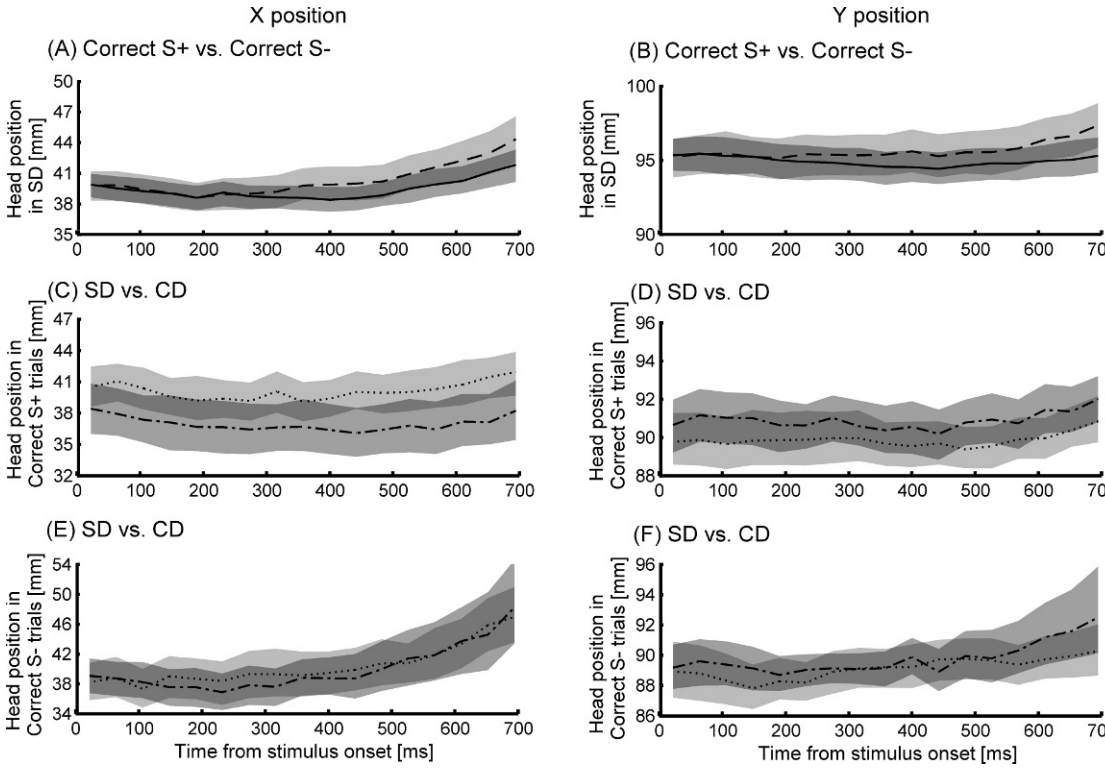

Fig. 9. Mean head position during the stimulus sampling period of the simple discrimination task (both visual and olfactory sessions were included, N = 3 and N = 7, respectively). Graphs show mean head position for Correct S+ and Correct S− trials in SD tasks (A, B; solid and dashed lines, respectively) and SD (dash-dotted line) versus CD (dotted line) in Correct S+ trials (C, D) and Correct S− trials (E, F). Mean head position was computed based on frame-to-frame positions of the center of the rat's head as estimated by the Cheetah Neuralynx system for videotracking LEDs on the rat's headstage (sampling rate: 25 frames/s). Grey bands flanking the mean-position curves indicate 95% confidence intervals, which were obtained by a bootstrapping method (Zoubir & Iskander, 2004). Dark grey areas reflect overlap in confidence intervals between Correct S+ and Correct S− trials or between SD and CD trials. X position (A, C and E) and Y position (B, D and F) are plotted as a function of time elapsed from stimulus onset. Although the head was not stationary during stimulus sampling, there was no significant difference between Correct S+ and Correct S− trials in SD, or between SD and CD studied for Correct S+ and Correct S− trials separately.

differ significantly between hit and correct rejection and SD versus CD trials (hit during SD: 4.24 ± 0.08 mm versus correct rejection during SD: 4.31 ± 0.12 mm; hit during CD: 4.15 ± 0.11 mm versus correct rejection during CD: 4.17 ± 0.20 mm; two-way ANOVA, $p > .05$). Likewise, no significant difference was found in the mean X and Y positions plotted as a function of time from stimulus onset (Figure 9). Despite the great similarities in head positions during hit and correct rejection trials, the graphs illustrate that the stimulus sampling period was not marked by a complete stasis of the head, but rather by a slight net movement on the order of a few millimeters. Thus, head movement was present, but in a relatively stereotyped, regular manner.

DISCUSSION

A behavioral setup was constructed with the aim of presenting a multimodal, hollow object to allow rats to sample sensory stimuli under conditions of strong behavioral regularity. Stimuli could be presented in an automated and temporally precise way, and the surrounding cage was equipped with a fluid port where rewarding (sucrose) or aversive stimuli (quinine solution) were delivered. Furthermore, the MMSC and surrounding cage permitted stable ensemble recordings from animals that

had been chronically implanted with an array of individually movable tetrodes, connecting to a sizable headstage (diameter: 5.6 cm) positioned above the rat's head. Although in this study we only trained rats to perform sensory-discrimination learning under undistracted (SD) or distracted (CD) conditions, the behavioral setup is useful to study a much wider range of cognitive processes, including attentional control, multisensory integration, working memory and sensorimotor control.

Considering that the associative learning procedures were completed by all 4 rats tested in this study and ensemble recordings were made from 2 rats, it can be concluded that the overall requirements set in the Introduction were largely met, although this conclusion deserves further comment. First, primary evidence for associative learning in a SD task with visual or olfactory stimuli was presented in Figures 5 and 6. Whereas the percentage of correct rejections can be regarded as a safe measure of discriminative operant conditioning in a task where animals produce Go responses by default, the difference in response latency for hits versus false alarms provided an additional measure of learning. That latencies for hits to visual stimuli became gradually shorter than for false alarms may be explained, on the one hand, by a strengthening of the stimulus–reward association and its utilization in hit trials, whereas on the other hand an increased latency in false alarm trials was likely coupled to an increased ability to withhold responding until, finally, this type of response was minimized altogether (Figure 6A).

Despite the observation that the same 4 rats were capable of visual as well as olfactory discrimination learning (Figure 5), it is interesting to note that all animals were slower in acquiring visual as compared to olfactory conditioning. Following initial SD acquisition, the odor also appeared to act as a stronger distractor than the visual stimulus, in the sense that task performance was more heavily disrupted upon the SD–CD transition in the visual task (Figure 7B) than in the olfactory task (Figure 7A). Although the serial position of these two tasks in the overall training schedule was different, this interpretation is supported by the fact that all rats were well above criterion before the distracting stimuli were introduced. That the rats were faster in acquiring olfactory discrimination relative to the visual task is well in agreement with the literature, although few studies (Brushfield, Luu, Callahan, & Gilbert, 2008) have directly compared learning in both modalities within the same animals (for olfactory discrimination: Eichenbaum, Shedlack, & Eckmann, 1980; Kay & Freeman, 1998; Sara, Roullet, & Przybyslawski, 1999; Schoenbaum & Eichenbaum, 1995; Tronel & Sara, 2002; van Duuren, et al., 2007; for visual discrimination: Bussey, Muir, & Robbins, 1994; Cook, Geller, Zhang, & Gowda, 2004; Markham, Butt, & Dougher, 1996; Minini & Jeffery, 2006; Simpson & Gaffan, 1999).

A further comment should be made concerning the requirement of temporal precision of stimulus delivery. On the one hand, the fast and reliable responding after reaching criterion demonstrates that animals were capable of appropriate stimulus sampling during the 700-ms presentation period, which implies that odor puffs were sufficiently discrete in both time and space to enable animals to perform olfactory conditioning efficiently. In this respect, the rats effectively functioned as ''biosensors'' for validating the systems for visual and olfactory presentation. On the other hand, this approach clearly sets limits to the extent that temporal precision of odor pulses can be claimed, whereas fast onset and offset of visual stimuli was reliably achieved using the masking method (see Method). To achieve trial-discrete odor presentation, our system was equipped with a dual-exhaust system consisting of a powerful, fast fan and a vacuum line, while a bypass system connected to the fan subserved rapid odor onset and largely avoided the problem of ''dead space'' (i.e. in between multiple valves for flow switching and the MMSC). These technical measures illustrate how odor application can be applied to larger chamber volumes on at least a trial-discrete basis.

Behavioral regularity during stimulus sampling is of great importance when one wishes to exclude motor confounds while examining neural correlates of stimulus processing, attention or related cognitive processes. First, execution of a relatively stereotyped sampling behavior was facilitated by the physical layout of the wall panel which required the animal to place its forepaws on a shelf below the head-entry port (Figure 2). Second, the behavioral setup was equipped with a system for video-

tracking head position by way of headstage LEDs. The mean travel distance of the head during stimulus sampling did not differ significantly during Correct S+ versus Correct S− trials during SD and CD. Furthermore, in the course of sampling, the mean X and Y positions of the head did not differ significantly between these trial types (Figure 9), even though these coordinates varied on average by a few millimeters during the sampling period. The videotracking system, relying on headstages that are attached to a cable and are situated approximately 5 cm above the animal's head, has a similar error margin. Altogether, these data indicate that a high degree of body–head regularity is achievable in rats processing sensory inputs.

The neural correlates observed during visual or olfactory SD performance pertained to all temporal phases of learning trials (stimulus, response, waiting, and reinforcement phases) and included subsets of stimulus-selective responses (Figure 8; whether this selectivity relates to feature tuning or motivational value remains to be determined). These results are in basic agreement with previous mPFC recordings studies in freely moving rats (Baeg, et al., 2003; Chang, Chen, Luo, Shi, & Woodward, 2002; Euston & McNaughton, 2006; Mulder, Nordquist, Orgut, & Pennartz, 2000; Pratt & Mizumori, 2001). Despite the variety of cognitive processes studied, a common denominator in mPFC recordings has been the broad coverage of relevant task components and phases by neural activity changes in mPFC. Although this patterning of neural activity may be explained by the notion derived from primate studies (Lauwereyns, et al., 2001; Rainer, Asaad, & Miller, 1998; Rao, Williams, & Goldman-Rakic, 2000) that the PFC has the capacity to filter out irrelevant information and focus on task-relevant events, this notion must be tested further. In this respect an advantage of the current behavioral setup is that a high degree of body–head regularity can be paired with the presentation of a multitude of stimuli from different modalities.

Apart from the experimental advantages touched upon above, the behavioral setup offers possibilities to study in rodents a multitude of cognitive tasks and their respective neurophysiological underpinnings. Using this technology, investigators may record large neuronal ensembles with single-unit resolution and combined with continuous local field potential measurements so that questions of neural synchrony, coherence and population coding can be addressed in a wide range of behavioral and cognitive conditions.

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